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Lymphotoxin β, a Novel Member of the TNF Family That Forms a Heteromeric Complex with Lymphotoxin on the Cell Surface

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Summary

The lymphokine tumor necrosis factor (TNF) has a well-defined role as an inducer of inflammatory responses; however, the function of the structurally related molecule lymphotoxin (LTα) is unknown. LTα is present on the surface of activated T, B, and LAK cells as a complex with a 33 kd glycoprotein, and cloning of the cDNA encoding the associated protein, called lymphotoxin β (LTβ), revealed it to be a type II membrane protein with significant homology to TNF, LTα, and the ligand for the CD40 receptor. The gene for LTβ was found next to the TNF-LT locus in the major histocompatibility complex (MHC), a region of the MHC with possible linkage to autoimmune disease. These observations raise the possibility that a surface LTα-LTβ complex may have a specific role in immune regulation distinct from the functions ascribed to TNF.

Introduction

The initiation of the immune response involves a complex array of intercellular signals, usually soluble cytokines coupled with a number of cell–cell contact-dependent signals. The contact-dependent events, most notably activation of the T cell receptor, lend specificity to the response, whereas the soluble mediators are generally responsible for maintenance of cell differentiation and proliferation. Tumor necrosis factor (TNF) and lymphotoxin (LT) (also called TNF-β) are related cytokines involved in many regulatory activities (Fiers, 1991; Beutler, 1990; Paul and Ruddle, 1988). Their roles in the immune system are somewhat of an enigma since in vivo experiments suggest very critical functions (Jacob and McDevitt, 1989; Ruddle et al., 1990; Kossodo et al., 1992), yet the corresponding in vitro work has not led to a very clear picture of their plac in T and B cell regulation (Tartaglia et al., 1991).

TNF is synthesized in response to various insults by a variety of cell types, including both hematopoietic and nonhematopoietic cells (Beutler, 1990; Spriggs t al., 1988; J vnikar et al., 1991), and is generally regarded as on of th primary initiating events in the inflammatory cascad . LT, in contrast, is made specifically by lymphocytes (Paul and Ruddle, 1988), and its biological role is not understood. Both genes lie closely spaced within the class III region of the major histocompatibility complex (MHC) (Spies et al., 1986; Nedospasov et al., 1986; Mueller et al., 1987; Gardner et al., 1987), yet they are clearly independently regulated (Sung et al., 1988). In general, LT and TNF display similar spectra of activities in in vitro systems, although LT is often less potent (Browning and Ribolini, 1989) or displays apparent partial agonist activity (Andrews et al., 1990). Moreover, the two known TNF receptors do not appear to discriminate between the two molecules (Schall et al., 1990; Smith et al., 1990). These observations suggested that LT was either a poorly redundant cytokine or that there were further facets, as yet unknown, to this cytokine. Within this context, it was of interest that LT is found on the surface of activated lymphocytes (Browning et al., 1991; Androlewicz et al., 1992; Ware et al., 1992; Abe et al., 1991, 1992; Miyake et al., 1992).

A number of what appeared originally to be soluble cytokines or growth factors have now been shown to exist in membrane-bound forms, e.g., transforming growth factor α, TNF, and the *kit* ligand (Massagué, 1990; Flanagan and Leder, 1990), and it is likely that the switching between soluble and membrane forms is an important regulatory event. In all of these cases, retention of a transmembrane region underlies the membrane association. TNF is a type II membrane protein similar to LT and the ligand for the CD40 receptor (Farrah and Smith, 1992; Hollenbaugh et al., 1992), and it is retained on the cell surface in both macrophages and T cells (Kreigler et al., 1988; Perez et al., 1990; Kinkhabwala et al., 1990; Ware et al., 1992). Surface LT does not result from the presence of the transmembrane region, but rather was found associated with a 33 kd integral membrane glycoprotein (Browning et al., 1991; Androlewicz et al., 1992). We hypothesized that this unique complex represented a more relevant form of LT and imparts specificity relative to TNF.

In this paper we describe the cloning of the gene encoding this second protein in the surface LT complex, note its resemblance to other members of the TNF–LT family, and delineate its genomic location next to the TNF–LT locus in the MHC. Since this protein, p33, forms a complex with LT, is structurally related to LT, and lies next to the TNF–LT locus in the genome, we have given the names LTα and LTβ to the original LT and the novel gene, respectively, as is typical of subunits of a single structure.

R sults

Isolation of th LTβ cDNA

The previously defined p33 protein was purified by affinity chromatography, and both N-t rminal and internal amino acid sequences were obtained. A d gen rate oligonucleotide based on the sequence EEEPET was used to screen a cDNA library from phorbol myristat acetate (PMA)-

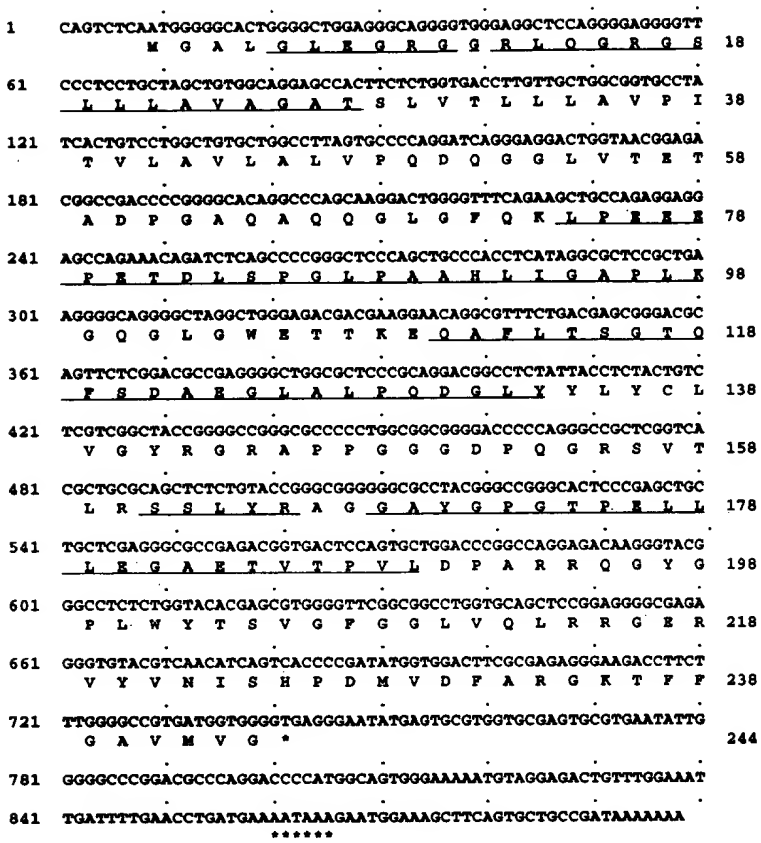

Figure 1. Nucleotide and Predicted Amino Acid Sequence of the cDNA Encoding LTβ

The putative membrane-spanning region is found between amino acids 19 and 48. The underlined sequences were previously determined by N-terminal amino acid sequencing of the affinity-purified protein and its tryptic fragments with spaces within one peptide, indicating a tentative assignment.

activated II-23 cells, a human T cell hybridoma that displays large amounts of surface LT upon phorbol ester activation (Browning et al., 1991). The cDNA encodes for a 240–244 amino acid sequence (molecular mass of 25–26 kd) typical of a type II membrane protein (Figure 1), and no identical sequences were found within the EMBL or GenBank data bases. This protein has been named LTβ (Figure 1). Following a short 15–19 amino acid N-terminal cytoplasmic domain, there is an extensive stretch of 30 hydrophobic amino acids that presumably acts as a membrane-anchoring domain. Biochemical analyses were consistent with the presence of one or more methionine residues within 10–20 amino acids from the N- or C-terminus and one or more cysteine residues (Browning et al., 1991). The cloned cDNA revealed the existence of one cysteine residue in the extracellular domain and two methionines within the last C-terminal 17 amino acids, in agreement with the prior characterization. The protein possesses an N-linked glycosylation consensus sequence, as previously expected on the basis of carbohydrate analysis (Browning et al., 1991). The difference between the 33 kd size of the previously analyzed protein and the encoded 25 kd results at least partially from N-linked glycosylation and possibly small inaccuracies in sizing on a SDS–polyacrylamide gel.

The 5′ end f the cDNA was difficult to d termine despite analysis of many ind pendent cl nes. Based on N-terminal amino acid analysis, the start site was believed to precede the GLEG sequence. The cDNAs uniformly lacked an ATG in the preceding codon yet did possess CTG in this position. It was postulated that a CTG translational start was utilized by this gene, and, as shown below, a cDNA clone starting at the first CTG was expressed in a transient transfection experiment in a functional form. To define further the 5′ cDNA sequence, primer extension analysis was undertaken, and 125–128 bp extension products were obtained (Figure 2) and sequenced. The complete cDNA sequence based on Maxam–Gilbert sequencing of the primer extension product reveals a 5–8 bp 5′ untranslated stretch and an in-frame methionine yielding a potential N-terminal amino acid sequence of MGALGLE. Overall, the difficulty in obtaining a full-length cDNA was due either to the very short nature of the 5′ untranslated end or the very GC-rich content of the 5′ end. Both leucines are encoded by CTG codons, and the proximity of the ATG to the 5′ end of the messenger RNA (mRNA) suggests that it may not be functional and that most translation initiates at one or both of the two CTG sites. No evidence for minor GALGLE or LGLE N-terminal amino acid sequences was found, which is consistent with a CTG start site. Moreover, the ATG codon lacks the typical consensus elements surrounding normal initiating codons, furth r implicating a CTG initiation sit (Kozak, 1986). If the CTG is the relevant initiating codon, the apparent lack of an N-terminal leucine suggests processing of whatev r amino acid is transf rred to this CTG. The exact nature of the prot in product arising from a CTG start codon in eukaryotes is unclear.

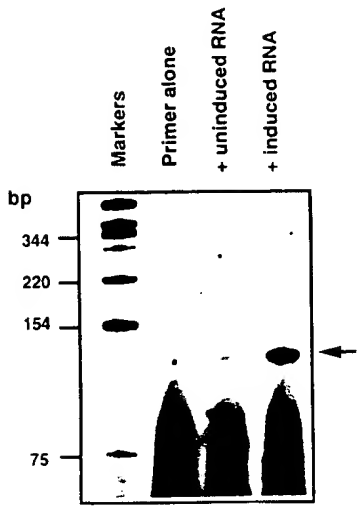

Figure 2. Primer Extension Analysis of LTβ RNA

Autoradiograph of a denaturing polyacrylamide gel of the extension products. Lane 1, labeled primer alone; lane 2, extension product using uninduced II-23 RNA; lane 3, extension product using PMA-induced II-23 RNA. The position of HinfI pBR322 fragments is indicated.

Comparison with the TNF Family

Comparison of the LTβ sequence with other proteins known to bind to members of the TNF receptor family reveals considerable structural similarity (Figure 3). All three known ligands to members of the TNF–nerve growth factor (NGF) receptor family (TNF, LTα, and the CD40 ligand) are type II membrane proteins and share at least five large regions of sequence conservation in the extracellular domain, as indicated in Figure 3. Using the alignment in this figure, LTβ is 21%, 24%, and 19% identical with TNF, LTα, and CD40 ligand, respectively, in the region defined by the fourth exon. This level of identity is basically similar to that found between TNF and LTα. The position of the glycosylation site in LTβ and CD40 ligand is identical but differs from the site in LTα. Both TNF and LTα are homotrimers, whereas the quaternary structure of the CD40 ligand is unknown. The regions of homology between members of the TNF family when in-laid into the crystal structure of LTα (Eck et al., 1992) are found primarily on the internal β strands A, H, C, and F, although the external-facing β strands A′ and G show significant conservation (Figures 4A and 4B). A view of the base of the LTα trimer reveals the conservation in A, F, C, and H strands where the interfaces form that stabilize the oligomeric structure (Figure 4C). The homology regions contain many of the contact residues involved in forming the trimer, except for three residues (51, 53, and 55) in the A′ strand that have conservative substitutions between LTα, LTβ, and TNF, but not CD40 ligand. Additional conserved residues (L102, W104, L113, L125, and L127) are located on outer β strands but point inward, contributing to the interactions between th int rnal and ext rnal sheets. Analysis of a space-filling model of LTα (Figures 4D and 4E) r veal that most of the conserved regions cr at a large contiguous swath of residues on the internal surface of the trimer. Only a small portion of the residu s in these homology regions are found at the solvent-acc ssible surface near the interaction crevice of the two subunits (except n ar the bas) where th r ceptors are thought to bind, and thus the noncons rved r sidues may impart receptor specificity to each ligand.

Characterization of the LTβ Gene

In light of the tandem arrangement of the TNF and LTα genes, a cosmid clone, O31A, containing the human TNF and LTα locus was examined and found to contain the LTβ gene. A 6 kb EcoRI fragment was sequenced and agre d with the primer extension sequence confirming the lack of an intron in the 5′ untranslated region (Figure 5). The exact location of the genomic EcoRI fragment was established by sequencing a XhoI fragment that linked the end of the EcoRI LTβ fragment and the 3′ end of TNF (Figure 6). The localization of the LTβ gene to within 2 kb of the TNF gen was not surprising in view of the proximity of the TNF and LTα genes (Nedwin et al., 1985). Thus, the LTβ gene is sandwiched between the TNF and *B144* genes in the class III region of the MHC. The *B144* gene is expressed in B cells and in macrophages (Tsuge et al., 1987) and had been found to be closely linked to the TNF–LT locus (Spi s et al., 1989). The LTβ gene is contained within four exons and spans 2 kb in an arrangement very similar to that of

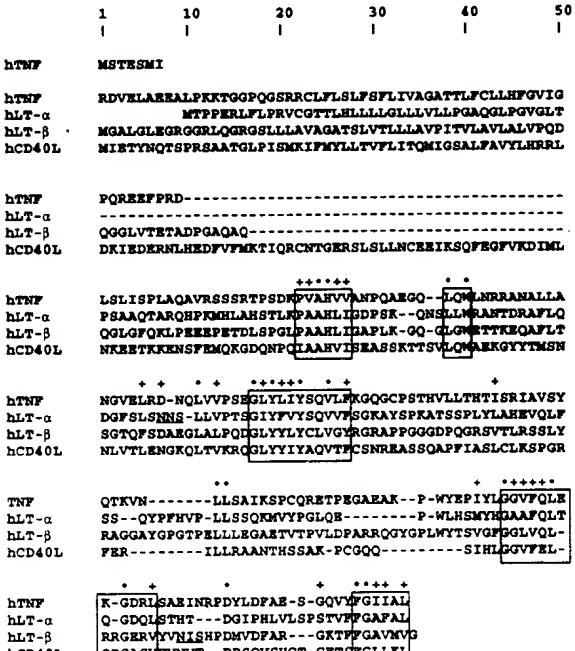

Figure 3. An Amino Acid Sequence Comparison of Four Members of the Family of Ligands Binding to Members of the TNF–NGF Receptor Family

Homology regions are shown boxed with sequence identity indicated by a dot and conserved sequences by a plus sign. Putative N-linked glycosylation sites are underlined. The sequence for human CD40 ligand was taken from Hollenbaugh et al. (1992).

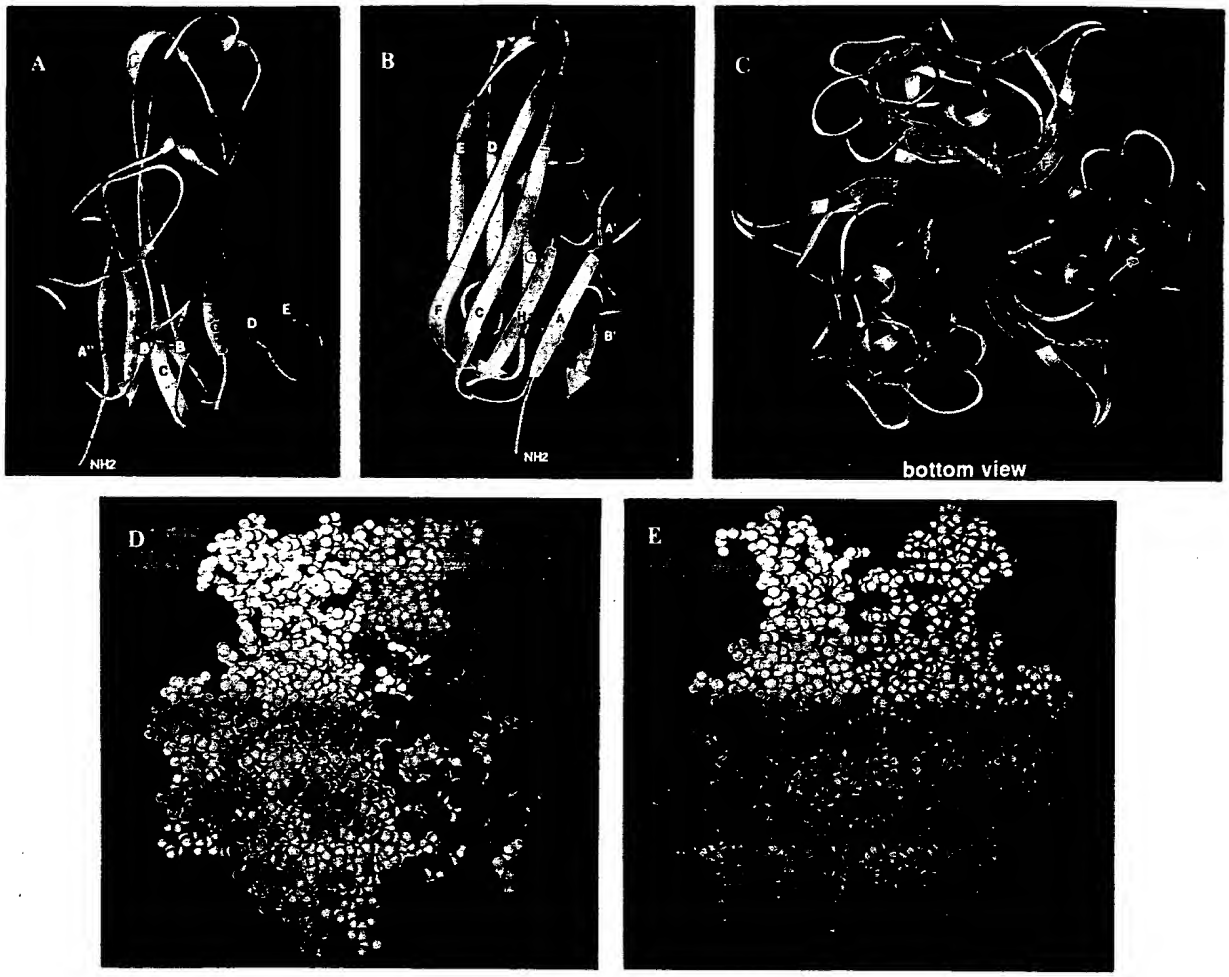

Figure 4. Homology Regions of the TNF–LT Cytokine Family Defined in the Crystal Structure of LTα

Individual β strands are labeled according to the convention of Sprang (Eck et al., 1992), where the A strand emerges from the amino-terminal end (residue 26) and the carboxyl terminus (residue 117) is at the end of the H strand. The conserved homology regions (HR) are defined, with the parentheses indicating the LTα sequence numbering and β strand assignment, as HR-1 (29–34; A), HR-2 (43–45; A'), HR-3 (71–81; C), HR-4 (136–147; F), and HR-5 (165–171; H) and correspond to the boxed residues in Figure 3. The homology regions are colored gold in all panels.

(A and B) A single subunit of LTα is shown (in schematic form using ribbons) in the orientation the monomer would have in the trimer with the amino terminus pointing downward (A) and the monomer rotated around the vertical axis, revealing the interior side of the subunit (B).

(C) A bottom view of the LTα trimer rotated 90° on the horizontal axis.

(D) A space-filling model of the trimer, in which the individual subunits are colored blue, green, or gray. The amino-terminal residues protrude from the base as shown above.

(E) The green-colored subunit has been removed, leaving a dimer that has been rotated around the vertical axis, exposing the interior.

TNF and LTα except for being oriented in the opposite direction. Only one copy of the gene is present in the human genome, as defined by stringent Southern analysis. The position of the intron–exon junction linking the last large exon that encodes essentially all of the extracellular domain and most likely the receptor-binding region is completely conserved in all three genes, underscoring the importance of the fourth exon. The promoter region of LTβ contains putative TATA and CAAT elements at positions −26 and −180. The AU-rich motif found in the 3′ untranslated region of LTα and TNF that is involved in determining mRNA stability is lacking in LTβ (Shaw and Kamen, 1986).

Expression of LTβ

Northern analysis of II-23 cells showed hybridization of the LTβ cDNA to a 0.9–1.0 kb mRNA, indicating that the cloned cDNA represents essentially all of the transcribed gene. The LTβ gene was expressed at low levels in untreated II-23 hybridoma cells; however, upon cell activation with phorbol ester, mRNA levels increased dramatically (Figure 7A). Thus, it is clear that expression of both subunits of the LT surface complex are induced upon activation of this T cell hybridoma. The cell line Hut-78, which constitutively displays surface LT (Ware et al., 1992), expressed low levels of LTβ mRNA in the absence of phorbol

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actggcttcctgcaagctaccttccctccctgggcctcagtttctctctctgctgagccagaagatgtctaaagacccctttggttccaccctgagagcc  100
tgtctccctaacctcaacttcttccccagttcagagaacccaggcatccagctgccccaccccagctctgggtaaacaggaagctgggtgaggggagcag  200
gggtgtgcggaaagtcccagccaggtgtgcaggtctacagggaggggtgggcccgtccctgaggtatgaaagccccctgctctggctctggTTCAGTCT  300
CAATGGGGGCACTGGGGCTGGAGGGCAGGGGTGGGAGGCTCCAGGGGAGGGGTTCCCTCCTGCTAGCTGTGGCAGGAGCCACTTCTCTGGTGACCTTGTT  400
  M  G  A  L  G  L  E  G  R  G  G  R  L  Q  G  R  G  S  L  L  L  A  V  A  G  A  T  S  L  V  T  L  L
GCTGGCGGTGCCTATCACTGTCCTGGCTGTGCTGGCCTTAGTGCCCCAGGATCAGGGAGGACTGgtgagtggctgcaacaggccctggtggagagttgta  500
 L  A  V  P  I  T  V  L  A  V  L  A  L  V  P  Q  D  Q  G  G  L
tcttgcggatgcttggctccctctggttgtgcctgtggtcttttgccccctctggctcagctggctcggctgtccctggtggggatgtcttgtctctttg  600
ctgactctctttccatgttcctgtgatgttgtgcttgtgtcccgacataagccccttgtgtctcctctcctcttcccgaggtacatctgtttctccgccc  700
aagtacctatgccttgcttgttctccctctaaggaggtgtgtgttggggatggtgctggtaggagaaaccccaggcctgcagcttgggtccactttcag  800
aggggtaggggtgacatgagctgaatctgaactctgggcactgtgaccccacccaaccagGTAACGGAGACGGCCGACCCCGGGGCACAGGCCCAGCAAG  900
                                                          V  T  E  T  A  D  P  G  A  Q  A  Q  Q  G
GACTGGgtaagagcagactgtctctccttcccgcttcagaccctcaggggctcccagctccctgctgcgtccccagatacctcttcctctaggaatcca 1000
  L  G
ggctccccatccctgcgccctgttctctcaagggtagcctgcatgggtggctgccctgcccccaatcgtggactctttgccccttccagGGTTTCAGAAG 1100
                                                                                          F  Q  K
CTGCCAGAGGAGGAGCCAGAAACAGATCTCAGCCCCGGGCTCCCAGCTGCCCACCTCATAGgtaaggacctccaagacctgaataagagtgtaaataatc 1200
L  P  E  E  E  P  E  T  D  L  S  P  G  L  P  A  A  H  L  I  G
cgaaggttccagttctgctcgcccagagtccttcggctccatgattccagtgctcggtttcccacccgcttcacgacctttgtcgctcgtgcccactct 1300
tacgctcgtcccccgcagtgtagtttcttcttccctccggtgcaagcaaaagccggcctggaggtccccactacagcgttctgcaccccacatccgtgttc 1400
cctcggccccaactcgcactcatcccagaaacagcaccatcccctcccccggcccggctcggctcccgcaggggctaaaagccgccacttccccaga 1500
agtcccaagcctttaggatcgcattcccaagagcgcgtcggcccgtgtctccgcagGCGCTCCGCTGAAGGGGCAGGGGCTAGGCTGGGAGACGACGAAG 1600
                                                          A  P  L  K  G  Q  G  L  G  W  E  T  T  K
GAACAGGCGTTTCTGACGAGCGGGACGCAGTTCTCGGACGCCGAGGGGCTGGCGCTCCCGCAGGACGGCCTCTATTACCTCTACTGTCTCGTCGGCTACC 1700
E  Q  A  F  L  T  S  G  T  Q  F  S  D  A  E  G  L  A  L  P  Q  D  G  L  Y  Y  L  Y  C  L  V  G  Y  R
GGGGCCGGGCGCCCCCTGGCGGCGGGGACCCCCAGGGCCGCTCGGTCACGCTGCGCAGCTCTCTGTACCGGGCGGGGGGCGCCTACGGGCCGGGCACTCC 1800
  G  R  A  P  P  G  G  G  D  P  Q  G  R  S  V  T  L  R  S  S  L  Y  R  A  G  G  A  Y  G  P  G  T  P
CGAGCTGCTGCTCGAGGGCGCCGAGACGGTGACTCCAGTGCTGGACCCGGCCAGGAGACAAGGGTACGGGCCTCTCTGGTACACGAGCGTGGGGTTCGGC 1900
 E  L  L  L  E  G  A  E  T  V  T  P  V  L  D  P  A  R  R  Q  G  Y  G  P  L  W  Y  T  S  V  G  F  G
GGCCTGGTGCAGCTCCGGAGGGGCGAGAGGGTGTACGTCAACATCAGTCACCCCGATATGGTGGACTTCGCGAGAGGGAAGACCTTCTTTGGGGCCGTGA 2000
G  L  V  Q  L  R  R  G  E  R  V  Y  V  N  I  S  H  P  D  M  V  D  F  A  R  G  K  T  F  F  G  A  V  M
TGGTGGGGTGAGGGAATATGAGTGCGTGGTGCGAGTGCGTGAATATTGGGGGCCCGGACGCCCAGGACCCCATGGCAGTGGGAAAAATGTAGGAGACTGT 2100
 V  G  *
TTGGAAATTGATTTTGAACCTGATGAAAATAAAGAATGGAAAGCTTCAGTGCTGCCGATAAAgatgctgagttgcgacacacgtcttaattcagggtggg 2200
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Figure 5. Genomic Sequence of Human LTβ

Introns are indicated by lowercase type. Putative TATA and CAAT elements in the promoter region are underlined.

stimulation. Molt-4 and THP-1 cells that do not display surface LT also do not express LTα or LTβ mRNA. The time course of phorbol ester induction of the LT subunits in II-23 cells was slightly different for LTα and LTβ (Figure 7B), suggesting nonidentical regulation, although whether these differences stem from differences in the promotor regions or in mRNA stability was not investigated.

Human peripheral blood lymphocytes (PBLs) cultured with anti-CD3 or interleukin-2 (IL-2) expressed both LT mRNAs (Figure 8), confirming earlier observations (Abe et al., 1992; Ware et al., 1992) that surface LT was found on activated lymphocytes. While there was variable loading of RNA in this analysis, comparison of RNA from fresh PBLs and from IL-2-activated PBLs clearly indicates induction of mRNA by IL-2 treatment. This observation correlates well with the abundant expression of surface LT on lymphokine-activated killer cells (Abe et al., 1992; Ware et al., 1992). Interestingly, there was some expression of LTβ mRNA in both freshly isolated PBLs and in resting cells after 24 hr in culture. Earlier fluorescence-activated cell

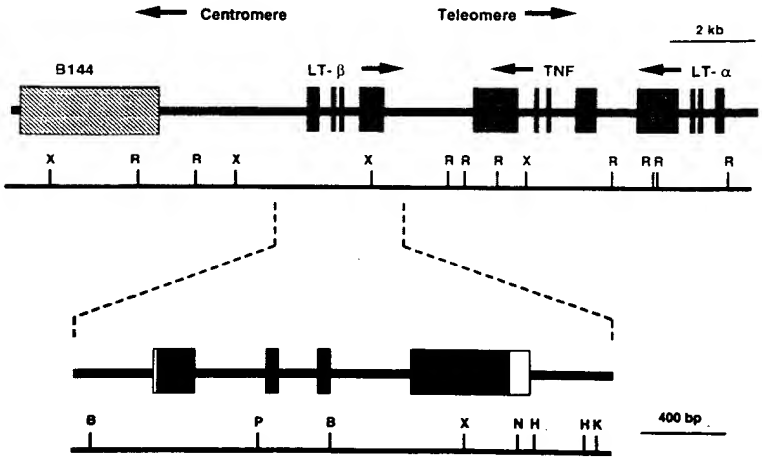

Figure 6. Schematic Diagram of the Region of Chromosome 6 Encompassing the TNF–LT Locus

The restriction map shows sites for EcoRI (E), XhoI (X), and HindIII (H) as determined by Nedospasov et al. (1986) and Spies et al. (1989) and confirmed in this work. The expanded region of LTβ shows restriction sites for BglII (B), KpnI (K), PstI (P), and NcoI (N).

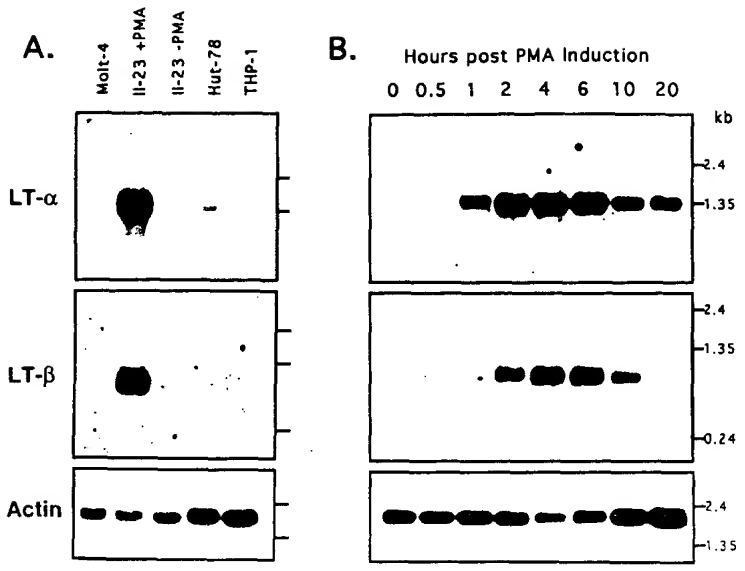

Figure 7. Northern Analysis of LTα and LTβ Expression in Several Cell Lines

(A) Specific expression of both LT genes in Hut-78 and II-23 cells.

(B) Time course of PMA induction of LT mRNAs in II-23 cells.

sorting (FACS) experiments also indicated low levels of surface LT in freshly isolated unstimulated cells that varied from donor to donor. LTβ mRNA was observed in the spleen and thymus, but not in lung, fetal or adult brain, heart, muscle, liver, kidney, or placenta, consistent with localization to lymphocytes (data not shown). Thus, these experiments suggest that LTα and LTβ expression may parallel each other, in agreement with the observation that all cell types known to produce LTα and LTβ also display surface LT (Ware et al., 1992). The B lymphoblastoid line RPMI 1788 does not display appreciable surface LT (Ware et al., 1992), yet it secretes LTα well, raising the speculation that there may be poor expression of LTβ and hence little diversion of LTα molecules to the cell surface.

To test the hypothesis that LTβ expression targets normally secreted LTα to the cell surface, CHO cells constitutively secreting LTα were transiently transfected with clone 12 LTβ cDNA. Surface LTα expression was assayed by staining with monoclonal anti-LTα, followed by FACS analysis. Transfection with LTβ led to surface LTα staining (Figure 9A), whereas a LTβ cDNA that contained a splicing error (clone 4), resulting in a nonfunctional frameshift, was unable to target LTα to the surface. Transfection of CHO cells not expressing LTα did not result in surface LTα expression, confirming that this monoclonal antibody cannot recognize the related LTβ protein (Androlewicz et al., 1992). An experiment with COS cells transfected with LTβ alone or cotransfected with LTα and LTβ cDNAs confirmed that surface LT expression requires both genes (Figure 9B). The cDNA clone used in these transfection experiments contained two base pairs between the cloning linker and the first CTG prior to the postulated GLEG mature N-terminal sequence. This DNA can encode for protein expression, and, given the lack of an upstream methionine in this clone, at least one of the putative initiating CTGs can be fully functional, as has been demonstrated for a small number of mostly regulatory proteins (Kozak, 1991). Subsequent experiments wherein the CTG was substituted with an ATG did not improve expression in a transient transfection (C. H., unpublished data).

Discussion

The cloning of LTβ and its ability to target LTα to the cell surface confirms our previous supposition that surface LT was a result of a heteromeric complex formed between the normally secreted LTα and the earlier defined p33

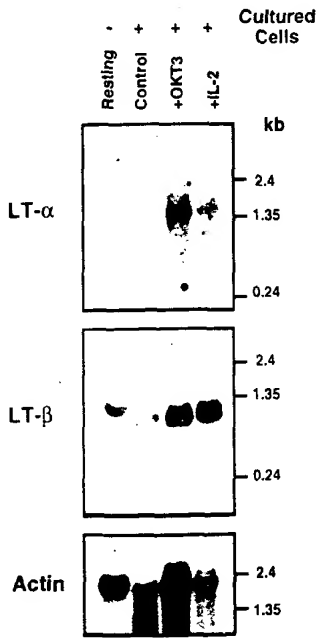

Figure 8. Northern Analysis

Northern analysis of fresh human PBLs and PBLs cultured for 1 day with media alone, anti-CD3, or IL-2.

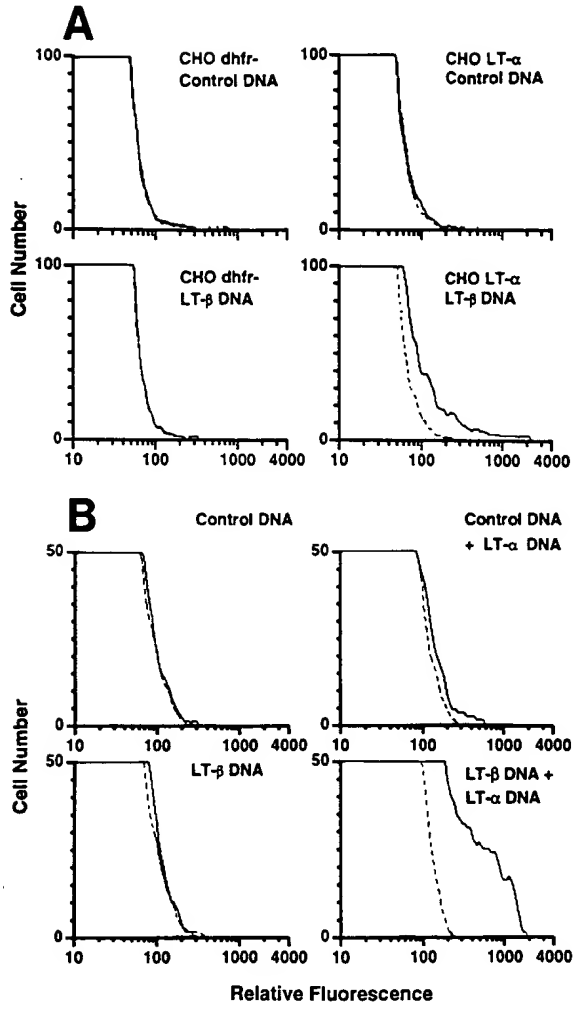

Figure 9. Expression of Surface LTα CHO and COS Cells in Transient Transfection Experiments

(A) CHO cells either dihydrofolate reductase minus (dhfr) or stably transfected with the LTα gene were transfected with either a control cDNA or the LTβ in pCDM8. After 2.5 days, cells were analyzed by FACS for surface LTα. Broken lines indicate control immunoglobulin G_1 staining, with solid lines showing anti-LTα staining.

(B) COS cells were transfected with control cDNA or LTβ with or without LTα cDNA in pCDM8 and analyzed as above.

molecule. The comparison of all three genes (TNF, LTα, and LTβ) led to the definition of homology regions, as previously described for the related CD40 ligand (Farrah and Smith, 1992; Hollenbaugh et al., 1992). TNF and LTα are known to be homotrimers (Wingfield et al., 1987; Smith and Baglioni, 1987; Browning and Ribolini, 1989), and the homology regions lie on the internal surfaces involved in trimer formation in th TNF and LTα crystal structures (Eck t al., 1992; Eck and Sprang, 1989; Jon s et al., 1989; Tavernier t al., 1989). This observation provid s a structural basis for the association b tw n LTα and LTβ in that this heterom ric complex most likely retains a trimeric structure similar to TNF and LTα, with th homology regions interacting in a het rotypic fashion. From chemical cross-linking exp rim nts with the het romeric surface LTα–LTβ complex, LTβ was believed to exist primarily as a dimer, and hence the stoichiometry of the overall compl x was believed to be $\alpha_1:\beta_2$ (Androlewicz t al., 1992). Preliminary data suggest that a small portion of the complex may also exist in an $\alpha_2:\beta_1$ ratio (C. F. W., unpublished data). Immunoprecipitation analysis indicated that LTα–LTβ complexes were not secreted (Browning et al., 1991); however, whether LTβ homooligomers are formed and secreted will be addressed with anti-LTβ monoclonal antibodies.

The concept of cell surface ligands as cytokines or growth factors has gained recognition primarily through the characterization of transforming growth factor α, TNF, and the *kit* ligand (Massagué, 1990; Flanagan and Leder, 1990; Wong et al., 1989), although membrane forms of a number of factors have been described (Massagué, 1990). The membrane form of LTα we have characterized differs from the above systems in that it does not retain its transmembrane domain, but rather is anchored via LTβ. The existence of a heteromeric complex of lymphokines is also reminiscent of signaling molecules in other areas, e.g., cytotoxic lymphocyte maturation factor (Gubler et al., 1991), platelet-derived growth factor (Raines et al., 1990), and heteromeric inhibin–activin complexes (Vale et al., 1990). The existence of similar heteromeric configurations of signaling molecules should be considered for other members of the TNF family. The elucidation of heteromeric receptor forms may be especially important for heteromeric ligands, as is the case for another member of the TNF receptor family, the low affinity NGF receptor that interacts with the *trk* proto-oncogene (Hempstead et al., 1991).

The restricted expression of LTα relative to TNF has tantalized workers in the field with the idea that LT has specific and important immunoregulatory functions (Paul and Ruddle, 1988; Ruddle and Homer, 1988). Delineation of the LTα–LTβ complex poses the possibility of immunoregulatory activities unique to the complex that cannot be mimicked by the LTα homotrimer. We are hypothesizing that the surface LTα–LTβ complex binds to a unique receptor or combinations of receptors, leading to a high affinity interaction and biologically relevant signaling. In support, preliminary data indicate that the major LT surface complex cannot bind to the two known TNF receptors (C. F. W., unpublished data). In this model, it is possible that the relatively poor activity of the LTα homotrimer relative to TNF in many systems indicates that the secreted LT phenomenon is only peripherally related to the true function of LT. The tethering of soluble LTα to the cell surface via complexation with LTβ raises the speculation that cell–cell contact-specific signaling through LTα–LTβ is an important aspect of immune regulation. The CD40 receptor–ligand pair represents a signaling m chanism whereby th T cell provides "h lp" to th B cell via a c ll–c ll contact (Armitag et al., 1992; Hollenbaugh et al., 1992). By drawing parallels with the CD40 system, on could postulate that surface LTα–LTβ may b a component of the regulation of T cells or other immune cells such as LAK or NK cells and B cells. Alternatively, in keeping with the known

cytotoxic activities of TNF and LTα, either LTβ or th LTα–LTβ compl x may be involved in inducing programm d cell death through a cell–cell contact-dependent mechanism. The programm d cell death obs rv d in conjunction with human immunodeficiency virus (HIV) infection may involve aspects of the LT system (Ameisen, 1992).

The TNF receptor family has grown to a substantial size, inviting the speculation that several TNF-like ligands may exist. The addition of the CD40 ligand, and now LTβ, to the family of TNF-related ligands reinforces this premise. The localization of the LTα gene to the TNF–LT locus would suggest more extensive duplication of a primordial LT gene than was previously realized, and several TNF-related ligands could be clustered within the class III region of the MHC. This region of the MHC may be associated with some autoimmune conditions such as insulin-dependent diabetes mellitus (Badenhoop et al., 1990; Porciot et al., 1991), and the potential disease linkage has inspired several analyses of gene structure in the region. Aberrant regulation of TNF has been proposed to be involved with the phenotype of the autoimmune NZB mouse (Jacob and McDevitt, 1989; Jongeneel et al., 1990), and chronic TNF administration can rescue the nonobese diabetic mouse (Jacob et al., 1990). In humans, TNF restriction-length fragment polymorphisms have been linked to various populations (Messer et al., 1991; Fugger et al., 1989; Dawkins et al., 1989; Webb and Chaplin, 1990), and these analyses can now be extended to this novel member of the region. Microsatellite DNAs have been described on both flanks of the TNF–LT locus; however, neither of the reported regions was located within the LTβ gene (Nedospasov et al., 1991). Given the localization of LTβ to this region, it is possible that this gene or its receptor is dysfunctional in certain autoimmune diseases. Interestingly, the Fas receptor is a member of the TNF–NGF family of receptors, and antibody binding to this protein can induce apoptosis (Itoh et al., 1991). A defective Fas receptor molecule in mice harboring the *lpr* allele results in a lymphoproliferative lupus-like disorder (Wantanabe-Fukunaga et al., 1992). Whether LTβ or the LTα–LTβ complex interacts with Fas or other orphan receptors in this family (Mallett and Barclay, 1991) can be readily addressed. These observations point to a fundamental role for this family of receptors and ligands in immune regulation. Now with delineation of LTβ and the CD40 ligand, it is clear that a family of TNF-related ligands is emerging to complement the already extensive family of TNF–NGF-type receptors. These receptor–ligand interactions point toward an additional array of important regulatory elements within the immune system, overlaying the known regulatory cytokine systems.

Experimental Procedures

Amino Acid Sequencing of LTβ

Il-23.D7 cells (5×10^{10}) were stimulated for 6 hr with 50 ng/ml PMA and lysed by nitrogen cavitation under conditions described previously, and LTβ was purified by affinity chromatography essentially as described previously (Browning et al., 1991). From the column eluate, roughly 2 μg was electrophoresed on a SDS–polyacrylamide gel and blotted onto ProBlott (Applied Biosystems), and the remaining 4–5 μg of material was similarly resolved and blotted onto nitrocellulose. The ProBlott was subjected to N-terminal amino acid sequencing (Matsudaira, 1987) using gas phase techniques in an automatic sequencer (Applied Biosystems). The nitrocellulose blot slice was digested with trypsin in situ as described (Aebersold et al., 1987), and tryptic fragments were resolved by narrow bore reverse-phase high pressure liquid chromatography and sequenced using liquid pulse sequencing.

Cloning of the LTβ cDNA and Genomic Fragment

A 32-fold degenerate oligonucleotide, GTYTCNGGCTCYTCYTC, was designed on the basis of the EEEPET sequence and used to probe a cDNA library prepared in pCDM8 with poly(A)$^+$ RNA from Il-23.D7 cells stimulated with PMA for 4 hr as described (Aruffo and Seed, 1987). Filters were washed with 3 M tetramethylammonium chloride at 50°C. Multiple clones were isolated and sequenced using dideoxynucleotide methodology. To isolate the LTβ gene, a BstEII–XmnI fragment of the cDNA was used to probe a Southern blot of the cosmid O31A provided by T. Spies (Spies et al., 1989). A 6 kb EcoRI fragment that cross-hybridized to the cDNA probe was subcloned into pNN109 (a derivative of pUC carrying a kanamycin-resistance gene) and sequenced. Separate XhoI fragments of this region of the cosmid were also subcloned into pBluescript II and sequenced.

Northern Analyses

Total RNA was isolated by the guanidine–SDS–cesium pellet method, or poly(A)$^+$ RNA was prepared with a Micro Fast Track kit (Invitrogen). Several human poly(A)$^+$ RNA samples were purchased from Clontech. Blots were prepared from formaldehyde–agarose gels and probed with a 0.6 kb BstEII–XmnI LTβ fragment, a 0.6 kb BamHI–ScaI fragment of human LTα, or a fragment of β-actin. Blots were hybridized at 65°C in a modified Denhardt's solution and washed with 0.5× SSC, 1% SDS at 65°C. Human PBLs were isolated using Ficoll and cultured in RPMI 1640 with 10% fetal bovine serum, glutamine, and antibiotics with or without 100 ng/ml IL-2 or 10 ng/ml OKT3.

Transient Expression of LTβ

CHO cells either dihydrofolate reductase minus or stably transfected with the LTα gene as described (Browning and Ribolini, 1989; the cell line was gift from Dr. W. Fiers) were transfected by electroporation, removed with Ca/Mg-free Hanks' solution with 5 mM EDTA, and analyzed by FACS. Cells were stained with 10 μg/ml of a monoclonal human LTα antibody (Boehringer Mannheim) followed by an affinity-purified Fab_2 goat anti-mouse fluorescein isothiocyanate–labeled antibody (Cappel). Cells were also stained with propidium iodine to allow exclusion of any dead cells. Cells were analyzed after 2.5 days with FACStar Plus (Becton-Dickinson), and because of the low expression levels in CHO cells, only the relatively bright fluorescein isothiocyanate cells were live gated. The data presented show only propidium-negative cells.

COS cells were transfected similarly either with 20 μg of clone 12 LTβ cDNA, a complete clone starting at the first CTG, or with control DNA that was clone 4, a LTβ cDNA with an internal frameshift due to a missplicing event. Both DNAs were transfected either alone or in conjunction with 20 μg of a complete LTα cDNA clone in pCDM8, previously isolated from the Il-23 cDNA library (M. Ward and J. L. B., unpublished data). The COS cell data were obtained from 30,000 events where only dead cells were excluded by a live gate.

Primer Extension Analyses

An oligonucleotide primer, GACAGTGATAGGCACCGCCAGCAACAA, was annealed to 20 μg of poly(A)$^+$ RNA from PMA-stimulated Il-23 cells for 6 hr at 65°C, precipitated, and extended at 37°C as described (Wallner et al., 1986). The extension products were resolved on a 6% polyacrylamide denaturing gel, and the extension product was excised and subjected to Maxam–Gilbert sequencing.

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GenBank Accession Numbers

The accession numbers for the sequences reported in this paper are L11015 (human LTβ cDNA) and L11016 (human genomic EcoRI fragment).